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(54) Title: ANALOGS OF PISCINE LHRH

(57) Abstract

An analog of piscine GnRH is disclosed having the amino acid sequence: pGlu-His-Trp-Ser-Tyr-W-Trp-Leu-Arg-Pro-Gly-X-Y-Z wherein W represents Gly or a D-amino acid, X represents a bond, or one or more amino acids which may be the same or different, Y represents Cys or Tyr or is absent, and Z is absent or represents one or more amino acids which may be the same or different or a continuous or discontinuous peptide chain, the or each discontinuity being a non-α amino acid polyfunctional linker moiety or a retro-inverso amino acid with the proviso that when W is Gly and X and Y are absent Z is present. The analog may be conjugated to a carrier and used, for example, to delay onset of sexual maturation and enhance growth.

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ANALOGS OF PISCINE LHRH

The present invention relates to extended analogues of the fish hormone referred to as piscine gonadotropin releasing hormone (pGnRH) and sometimes called piscine luteinizing hormone-releasing hormone (pLHRH). In particular, it relates to analogues of pGnRH formed by adding to the C-terminus of the native pGnRH amino acid sequence a short amino acid sequence comprising a cysteine or tyrosine residue, such that the conformation in solution is substantially unchanged, polypeptide conjugates thereof suitable for raising anti-pGnRH antibodies and methods of immunising fish.

Commercial fish farming is a rapidly growing industry worldwide. Western Europe, North and South America, Canada and the Eastern Seaboard are all major fish producers, notably of salmon. For example, the USA produced 185,000 tonnes of salmon in 1989, while their catfish market followed closely behind. Norway, on the other hand, the major producers of Atlantic Salmon in Western Europe, produced 120,000 tonnes in 1989/90. It has been calculated that the world market in salmon is set to grow by an average of 7.5% every year until the end of the century and result in a turnover worth £2000 million per annum. Commercial development of sea bass and turbot, and in the longer term of halibut and cod, is envisaged while at present Europe is the major producer of sea bream and sea bass with carp being farmed in both Eastern Europe and Africa.

In aquaculture there is a great need to control sexual maturation of fish in order to prevent the following: diversion of somatic growth into the gonads; loss of flesh quality; and appearance of commercially undesirable secondary sexual characteristics. This is especially true of salmonids, where sexual maturation of

precocious parr and grilse (usually immature stages of development) can have severe economic effects by arresting growth of parr at a non-commercial size, and forcing the harvesting of grilse over a short period thereby lowering market value. In addition, there is a desire to inhibit fertility of farmed fish in case they stray from farms and contribute to natural gene pools.

Logistical problems are associated with harvesting sexually mature fish. Such fish cannot be sold and invariably a certain percentage of fish do mature before others, males having the predominant tendency for precocious maturation. Separating sexually mature fish from immature fish is a time consuming and expensive process. Once fish start maturing, they do so at more or less the same time and rate, which makes harvesting an enormously difficult task. It follows that a method of controlling fish maturation in phases clearly would be of benefit.

The ideal weight for harvesting salmon, for example, is often not achieved due to precocious maturation, thus forcing sub-optimal harvesting of fish. Controlling maturation would avoid sub-optimal harvesting and allow for complete optimization of size.

Hitherto the most effective way to ensure that sexual maturation does not occur is to rear sterile fish. Despite numerous experimental approaches this has not yet been achieved for populations showing sexual dimorphism.

Several methods have been used to control sexual maturation in fish including surgical castration, triploidization and sex control (the latter two of which are both genetic techniques). Attempts were made in the past to surgically castrate fish but they were abandoned due to the great expense incurred and the trauma to fish that followed such procedures.

Tripoloidy is a method giving rise to the presence of three sets of chromosomes in an individual which

induces sterility in both triploid male and female fish. However, there are several disadvantages associated with triploidy. Firstly, it is a tedious and time consuming process carried out over a long period. Eggs for triploidy have to be of high quality to survive the treatments necessary. Although the resulting fish seem to be fully viable, the survival rate includes a 15-20% average loss with the growth rate of triploid fish being slightly slower than that of diploid fish outside the spawning season. During the approach to spawning there is a big difference between male and female triploids in that males develop near normal testes and all associated secondary sexual characteristics while female triploids do not develop significant ovaries and have a juvenile appearance at natural spawning time.

Successful application of induced triploidy requires it to be combined with a female-only technique, which is not commercially economic. Further disadvantages associated with triploids are that they do not grow well in competition with diploids in the same tank, and triploid females tend to show no superiority in growth over maturing diploid females.

Fish are highly dimorphic, i.e. they can interchange from male to female and vice versa, and the change of sex can be controlled. One sex is usually more preferable for cultivation purposes, for example from the viewpoint of growth rate. Turbot and eel are examples where the females grow much bigger than males. In salmonid species, juvenile males and females grow at about the same rate, but males develop undesirable features later. At maturation, males cease to grow, become discoloured and aggressive and are of very poor value either as food or sport. Also, maturing males of reared rainbow trout and Atlantic salmon species, once put into sea-water culture, are unable to make the necessary adjustments to salt water and die. A method of sex reversal to obtain all female populations can be

applied, but it involves incorporation of hormones into fish feeds, making such fish unsuitable as food for the table. Other approaches to control fish fertility such as irradiation, chemosterilization and hormone administration, have so far proved unreliable, uneconomical or unsuitable for use on fish destined for the table.

Immunological methods of controlling sexual maturation have shown some success in mammals. However, studies of mammalian hormonal and immunological responses cannot form a firm basis for investigation of piscine physiology. Fish are cold blooded and their immune system varies under different conditions. Temperature has a marked effect on the immune response in fish, so that the higher the temperature within the physiological range, the higher the level of the immune response. Physiologically low temperatures retard the rate of antibody production and may inhibit the establishment of immune memory, where T lymphocytes appear to be particularly sensitive.

In addition, the immune system of fish is influenced by hormones which may vary seasonally. Hormone levels are modified by stress from handling and transfer of fish, or poor water quality such as low oxygen content. During smoltification and sexual maturation, both periods of profound hormonal changes, fish are more susceptible to disease, due to a reduction in the number of lymphocytes.

Furthermore, fish only produce one type of antibody molecule, IgM, whereas five main classes of antibody have been identified in mammals.

Also, the endocrine system of fish is substantially different to that of mammals. For example piscine gonadotropin-releasing hormone, which is produced by salmonids such as salmon and trout for example, has the sequence:

pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH2

(Sherwood et al, 1983; "Characterisation of a teleost GnRH", PNAS USA 80: 2794-2798) which differs at positions 7 and 8 when compared to mammalian LHRH.

We have now discovered a class of analogues of piscine GnRH which can be conjugated to a suitable carrier molecule and used to immunise fish such that the antibodies elicited against the immunogen cross-react with high affinity and neutralise the endogenous GnRH.

According to one aspect of the present invention, we provide an analogue of piscine GnRH having the amino acid sequence:

pGlu-His-Trp-Ser-Tyr-W-Trp-Leu-Arg-Pro-Gly-X-Y-Z

wherein W represents Gly or a D-amino acid,

X represents a bond, or one or more amino acids which may be the same or different,

Y represents Cys or Tyr or is absent, and

Z is absent or represents one or more amino acids which may be the same or different or a continuous or discontinuous peptide chain, the or each discontinuity being a non-α amino acid polyfunctional linker moiety or a retro-inverso amino acid with the proviso that when W is Gly and X and Y are absent Z is present.

Preferably, the residue at position W is Gly, which is the naturally occurring residue, and the residue at position Y is advantageously Cys, because it offers a more specific and convenient conjugation to a carrier protein than can be obtained with a C-terminal Tyr.

It is preferred that X represents a relatively small number of residues, e.g. one to fifteen, preferably, one to eight, more preferably one to three and most preferably only one. Although any one or more amino acids may be present at position X, Gly is the most preferred amino acid residue. Of course, other amino acids, preferably with small side-chains such as Ala, Ser, Asn and Val, may be present at position X and any amino acid residue at this position is included

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within the scope of the invention. It should also be noted that where more than one residue is included at position X, the residues may be the same or different, such as in for example:

pGlu-His-Trp-Ser-Tyr-W-Trp-Leu-Arg-Pro-Gly-Gly-Ala-Y-Z.

It should be understood that pGnRH in which no amino acid residue occurs at position X are included within the scope of the present invention.

In preferred embodiments of pGnRH according to the invention, there is no amino acid residue at position Z and a particularly preferred embodiment of the analogue according to the invention has the following sequence:
pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Arg-Pro-Gly-Gly-Cys.

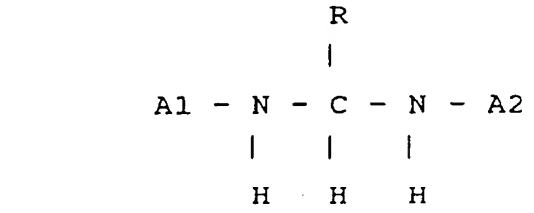
However, our studies have shown that inclusion of additional amino acids at position Z will generally have no significant effects on the conformational preferences of the remainder of the molecule. If such an extension at Z is included, then in preferred embodiments of the invention, the extension will include one or more segments of protein sequence with the ability to act as a T-cell epitope. For example, segments of amino acid sequence of the general formula 1-2-3-4, where 1 is Gly or a charged amino acid (e.g. Lys, His, Arg, Asp or Glu), 2 is a hydrophobic amino acid (e.g. Ile, Leu, Val, Met, Tyr, Phe, Trp, Ala), 3 is either a hydrophobic amino acid (as defined above) or an uncharged polar amino acid (e.g. Asn, Ser, Thr, Pro, Gln, Gly), and 4 is a polar amino acid (e.g. Lys, Arg, His, Glu, Asp, Asn, Gln, Ser, Thr, Pro), appear to act as T-cell epitopes in at least some instances (Rothbard, J.B. & Taylor, W.R. (1988). A sequence pattern in common to T-cell epitopes. The EMBO Journal 7(1): 93-100). Similarly segments can be of the sequence 1'-2'-3'-4'-5', wherein 1' is equivalent to 1 as defined earlier, 2' to 2, 3' and 4' to 3, and 5' to 4 (ibid). Both forms are included in a general form as defined below:
pGlu-His-Trp-Ser-Tyr-W-Trp-Leu-Arg-Pro-Gly-X-Y-S-T-S',

wherein T denotes a segment of peptide sequence

containing one or more T-cell epitopes (preferably less than five) which may be of the type defined in the previous paragraph or may be of other structure and which may be separated by spacer segments of any length or composition, and S and S' (either of which may optionally and independently be omitted) represent spacer segments of amino acid sequence of any length and any composition. Preferably, S comprises less than five amino acid residues in length and comprises for example residues selected from Gly, Ala, Pro, Asn, Thr, Ser or polyfunctional linkers such as non-α amino acids. S' is preferably less than ten amino acid residues and may include polyfunctional linkers such as non-α amino acids. The moieties of S' are preferably drawn from the same group as given for S above. It is possible for S' to represent a complete protein, thus obviating the need for conjugation to a carrier protein.

It should be noted that when Z is absent, the T-cell epitopes mentioned above may be provided at position X.

Also included within the scope of this invention are derivatives of the basic analogue in which Z is or includes a "retro-inverso" amino acid, i.e. a bifunctional amine having a functional group corresponding to an amino acid. For example an analogue according to the invention and containing a retro-inverso amino acid may have the formula:



where R is any functional group, preferably Gly, and A1 and A2 are preferably each a copy of one of the analogues defined herein (but not necessarily the same) attached by its C-terminal end. In particularly

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preferred embodiments, the Tyr or Cys at position Y in one or other of A1 or A2 should be replaced by any amino acid type other than the residue in the corresponding position of the other copy, or alternatively it may be omitted. Conjugation of this dimer is thus effected through the side chain of one or other (and optionally both) of the residues at position Y. Alternatively, A1 and/or A2 may be natural pGnRH and R of the retro-inverso amino acid may be Cys or Tyr. T-cell epitopes may optionally be included in segment Z as discussed earlier.

Analogues may have the formula:

$$A_1 - B - A_2$$

wherein B is a retro-inverso amino acid and A_1 and A_2 are the same or different and have the sequence:
pGlu-His-Trp-Ser-Tyr-W-Trp-Leu-Arg-Pro-Gly-X-Y-Z'
in which W, X and Y are as defined above and Z' is the same as Z defined above except that it does not include a retro-inverso amino acid.

Retro-inverso modification of peptides involves the reversal of one or more peptide bonds to create analogues more resistant than the original molecule to enzymatic degradation and offer one convenient route to the generation of branched immunogens which contain a high concentration of epitope for a medium to large immunogen. The use of these compounds in large-scale solution synthesis of retro-inverso analogues of short-chain biologically active peptides has great potential.

Another aspect of the invention provides a process for the manufacture of an analogue of piscine GnRH having the amino acid sequence:

pGlu-His-Trp-Ser-Tyr-W-Trp-Leu-Arg-Pro-Gly-X-Y-Z

wherein W represents Gly or a D-amino acid,

X represents a bond, or one or more amino acids which may be the same or different,

Y represents Cys or Tyr or is absent, and

Z is absent or represents one or more amino

acids which may be the same or different or a continuous or discontinuous peptide chain, the or each discontinuity being a non-α amino acid polyfunctional linker moiety or a retro-inverso amino acid with the proviso that when W is Gly and X and Y are absent Z is present, the process comprising the steps of coupling the residues using chemical, biological and/or recombinant techniques known per se and isolating the analogue.

Analogues according to the invention may be synthesised by standard peptide synthesis techniques. For example solid-phase peptide synthesis (SPPS) introduced by Merrifield in 1963 (J.Am.Chem.Soc. 85, 2149) may be used. In this approach, a growing peptide chain is bound covalently through its C-terminus to an insoluble solid support. Synthesis is carried out by the successive addition of amino acids in the desired sequence. All of the intermediate steps of purification, which are necessary for synthesis in solution, are reduced in this case to simple washings, since most of the side-products of reaction and degradation are dissolved in the reaction mixture. The advantages of SPPS are speed, the relative ease of implementation and the fact that it is a method which can be partially or completely automated. It is possible to produce several tens of grams of peptide using SPPS.

SPPS as developed by Merrifield in 1963 can be briefly described as comprising four stages:

(A) A first protected amino acid is linked to the solid support through a covalent bond which remains stable throughout the synthesis;

(B) A free amino group of the bound residue is regenerated by deprotection under conditions in which the protecting groups of any side chain functional

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groups are stable;

(C) The next protected amino acid is coupled with the first by condensation to give rise to the formation of an amide bond. The steps B and C are repeated until synthesis is complete;

(D) At the end of the synthesis, the peptide is released from the solid support and any amino acid side chain protecting groups are removed.

A considerable number of combinations of amino acid protecting groups have been described in the literature. However, at present, only two of these combinations are commonly used. The first is the tert-butoxycarbonyl (Boc)/benzyl combination which has been used by Merrifield since 1963 and which still remains the most widely used. More recently, the fluorenylmethoxycarbonyl (Fmoc)/tert-butyl system, developed by Sheppard et al has found increasingly wide application (Sheppard, R C, 1986. Science Tools. The LKB Instrument Journal 33, 9). Further examples include the Fmoc-polyamide method using the solid phase resin developed by Arsady, R. et al (J. Chem. Soc. Perkin Trans. 1981 1, 529-537), fluorenyl-methoxycarbonyl (Fmoc) protection of the individual amino acids incorporated (Atherton, E. et al, J. Chem. Soc. Perkin Trans. 1983 L 65-73) and 9-fluorenyl-methoxycarbonyl (Fmoc) Chemistry (see, for example, Atherton, E et al (1985) J. Chem. Soc. Chem. Comm.165).

Also encompassed within the scope of this invention are DNA and RNA molecules encoding, via the genetic code, the sequences of any of the peptide analogues described herein. Such DNA molecules may be used in a suitable microbiological or eukaryotic cell culture expression system to produce the analogues described herein. Similar molecules have been synthesised in such systems and are described in the literature (see for

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example European Patent Application No: 85307311.2).

It should be noted that analogues incorporating retro-inverso amino acid derivatives cannot be made directly using such a system. However, the basic analogues can, and they can then be purified and chemically linked to the retro-inverso amino-acids using standard peptide/organic chemistry. A practical and convenient novel procedure for the solid-phase synthesis on polyamide-type resin of retro-inverso peptides has been described recently [Gazerro, H., Pinori, M. & Verdini, A.S. (1990). A new general procedure for the solid-phase synthesis of retro-inverso peptides. In "Innovation and Perspectives in Solid Phase Synthesis" Ed. Roger Epton. SPCC (UK) Ltd, Birmingham, UK].

Preferably, the analogue is conjugated to a carrier molecule to give an analogue-conjugate. Any small- or macro-molecular carrier or microorganism may be used, but the following are preferred amongst conventional carriers: keyhole limpet haemocyanin (KLH), bovine serum albumin (BSA), chicken gammaglobulin (CGG), tetanus toxoid (TT), diphtheria toxoid (DPT), purified protein derivative of tuberculin (PPD), muramyl dipeptide (MDP), soybean trypsin inhibitor (STI), and cholera toxin or its B subunit. Note that priming with BCG vaccine may be advantageous in the case of PPD (Lachmann, P. et al., 1986. In "Synthetic Peptides as Antigens", Ciba Foundation Symposium 119 pp. 25-40).

Particularly preferred as carriers for the LHRH analogues when autoimmunising fish are the following micro-organisms or macro-molecular antigens therefrom which are components of fish vaccines: Aeromonas salmonicida (furunculosis), Yersinia ruckeri (ERM) and a mixture of Vibrio anguillarium and Vibrio ordalii (vibriosis).

Recently, five basic types of vaccine have been tested for protection against furunculosis which is caused by the Gram-negative non-motile bacterium

Aeromonas salmonicida: whole killed or disrupted cells (bacterins), extra cellular products (ECP) and ECP toxoids, whole killed cells in combination with ECP, live attenuated vaccines and purified antigens. In addition hyperimmune sera, to certain antigens, raised in fish or mammals have been used for passive protection. Also, a successful vaccine (or bacterin) against enteric redmouth (ERM) caused by the gram-negative motile bacterium Yersinia ruckeri has been produced which comprises formalin-inactivated whole bacterial cultures. Current commercial vibriosis vaccines in the Northern Hemisphere contain mixtures of the most common species, V.anguillarium and V.ordalii. These vaccines are simple inactivated cultures containing mixtures of whole cells and ECP.

A further aspect of the invention provides a vaccine comprising a pGnRH analogue or analogue conjugate, according to the invention, and a veterinarially acceptable excipient.

Such vaccines may be used to autoimmunise fish, and a further aspect of the present invention provides a method of inhibiting sexual development in fish which comprises administering pGnRH analogue or analogue-conjugate to said fish in a effective amount to produce a titre of anti-pGnRH antibodies sufficient to reduce significantly the biological efficacy of endogenous pGnRH.

Conjugation of analogues containing either Cys or Tyr at position Y may readily be made using reagents and procedures well described in the literature. For example, N-γ-maleimidobutyryloxysuccinimide (Calbiochem) may be used to conjugate via a thioether bond analogues of piscine GnRH containing a Cys-residue at position Y to lysine side-chains of the carrier protein, whilst a diazo bond may be formed with N-(4-diazophenyl)-maleimide for the conjugation of analogues containing a Tyr residue at position Y.

Peptides and conjugates can readily be purified to the necessary degree using methods well known in the art including, for example, high-performance liquid chromatography, sodium dodecyl sulphate-polyacrylamide gel electrophoresis and fast-performance liquid chromatography.

The peptide-carrier conjugate according to the present invention may be used for the following:-

1) Immunising any animal to raise anti-piscine-GnRH antibodies for uses specified below;

2) Autoimmunising fish to piscine GnRH in order to:
- slow, halt or regress sexual development
- control, limit or eliminate fertility
- treat GnRH-dependent piscine tumours
- carry out piscine physiological/veterinary research;

3) As a diagnostic agent for the presence of anti-piscine-GnRH antibodies in, for example, serum samples.

Anti-piscine-GnRH antibodies raised against the analogue also form part of the invention. They may be used for example:-

1) As research tools to explore piscine physiology/immunology;

2) As immunogens to raise anti-idiotype antibodies;

3) For passive immunisation in order to:
- achieve short-lived slowdown, halting or regression in sexual development
- accomplish short-lived control of fertility
- treat GnRH-dependent piscine tumours
- otherwise interfere with piscine GnRH activity.

(Passive immunisation may be effected through

administration of polyconal, monoclonal and single domain antibodies raised in fish or mammalian species) and

4) As diagnostics for the presence of piscine GnRH in, for example, serum samples.

It will be apparent that anti-idiotype antibodies mentioned at 2) above form part of the present invention.

The preparation of polyclonal or monoclonal antibodies, chimaeric and piscinised forms of such antibodies (see, for example, Morrison et al. (1984) PNAS (USA) 81, 6851-6855; Riechmann et al. (1988) Nature 332, 323-327), single domain antibodies (see, for example, Ward, E.S., Gussow, D., Griffiths, A.D., Jones, P. and Winter, G. (1989) Nature 341 544-546), which bind specifically to a synthetic polypeptide according to the present invention, may be carried out by conventional means and, as stated above, such antibodies are considered to form part of this invention.

In respect of detection of anti-piscine GnRH antibodies or piscine GnRH, the skilled person will be aware of a variety of immunoassay techniques known in the art, inter alia, sandwich assay, competitive and non-competitive assays and the use of direct and indirect labelling. Preferably, a kit for detecting anti-piscine GnRH antibodies will comprise an analogue or analogue conjugate according to the invention, label means and a solid support. Similarly, a kit for detecting pisicine GnRH will comprise an antibody or antigen binding fragment which specifically binds an analogue according to the invention, label means and a solid support.

Several methods exist for the administration of fish vaccines: e.g. by injection, through the oral route and via immersion.

Intraperitoneal injection is the most effective method of vaccination and furthermore it permits the use of adjuvants which enhance the magnitude of the immune response. Disadvantages are that fish require anaesthetization and handling which cause stress and is also very labour intensive. However, by using repeater syringes and a production line system, 1000 fish can be injected per hour. However, it cannot be used on fish much below 15 g.

Oral vaccination is suitable for mass administration to fish of all sizes and imposes no stress due to handling. However, intrinsic limitations exist whereby large amounts of vaccines are required, increasing cost and uncertainty of individual dosage. Oral vaccines have poor potency resulting in low or inconsistent levels of protection. Most oral administration work has been carried out with vaccines for vibriosis.

Direct Immersion (D.I.) which is preferred, is simple and rapid requiring only a few seconds of exposure to the vaccine. This method is now automated and a 'bath' or 'flush' variation was developed for vibriosis and ERM vaccines, and simply involves pouring the vaccine into holding tanks. Although, this method consumes more vaccine and requires longer exposure (about one hour), which involves oxygenation of the water and close monitoring of fish for stress, it is less labour intensive than injection.

It may be advantageous to immunise with a cocktail containing (i) a given analogue conjugated to more than one type of carrier molecule, and/or (ii) more than one kind of analogue conjugated to the same carrier molecule. Moreover, any of the peptide analogues, their conjugates, and cocktails thereof may be administered in any suitable adjuvant or delivery system, and more than one adjuvant or delivery system may be combined to form a so-called "super-cocktail". Preferred adjuvants and

delivery systems include aluminium hydroxide (alum), microspheres, liposomes, micelles, niosomes, ISCOMS, Brauns lipoprotein and whole-cell or components of microbial fish vaccines.

The invention will now be described by way of non-limiting examples.

Example 1

A carboxy extended form of pGnRH according to the invention is synthesised using standard solid-phase Fmoc methodologies. The peptide is cleaved from the resin in the presence of trifluoroacetic acid and subsequent purification is achieved by gel filtration, ion exchange chromatography and reverse phase high performance liquid chromatography. The peptide is conjugated to a variety of carriers by MBS (m-Maleimide benzoyl-N-hydroxysuccinimide ester).

Chicken gamma globulin (CGG) has already been shown to be a good carrier in rainbow trout for other vaccines and is used in this example. Also PPD is used since it is a proven carrier in mammalian studies (even though prior immunisation with BCG is required).

The pGnRH analogue is coupled to the carrier in a molar ratio of peptide:protein of 30-40:1 and three different concentrations injected intraperitoneally into rainbow trout.

The ensuing serum antibody response is monitored weekly for 10 weeks by enzyme immunoassay (ELISA) using Horseradish Peroxidase (HRP) conjugated monoclonal anti-trout Ig. In some tests in this example preimmunisation with carrier or the use of adjuvants or booster injections are effected in order to determine an optimal protocol for producing anti-pGnRH antibodies.

Example 2

The optimal protocol determined in Example 1 is used to immunise a group of potentially maturing rainbow

trout. Antibody response is monitored as in Example 1 together with monitoring of growth rate and the production of sex steroids (e.g. testosterone). At the end of the experiment the fish are killed, the gonads removed and weighed to determine the gonadosomatic index (GSI). The gonads are then fixed for later histological study by light microscopy. Immunised fish show a significantly better GSI than untreated control fish.

Example 3

Immunisation of maturing rainbow trout with salmonid gonadotropin-releasing hormone extended analogue coupled to tuberculin purified protein derivative (sGnRHa-PPD).

Methods

a) Conjugation of sGnRH-Gly-Cys to PPD

Tuburculin PPD (1.008 mg/ml + phenol preservative, Evans Limited) was dialysed overnight in benzyolated cellulose tubing against 0.9% NaCl at 12°C to remove the phenol. The PPD was then concentrated to 4.032 mg/ml with polyethylene glycol (PG 20000) for 1.5 hours. PPD solution (2.5 ml containing 10.08 mg PPD) was mixed for 1 hour at room temperature with 1.08 mg N-γ-maleimidobutyryloxysuccinimide (GMBS) in 5.04 µl dimethyl formamide (DMF) and then applied to a sephadex column G-25M PD-10 and eluted with 3.5 ml Buffer A (0.05M NaH_2PO_4, 0.14M NaCl, pH 7.0). Drop by drop 4.687 ml of 3 mg/ml sGnRH-Gly-Cys dissolved in distilled water then Buffer A (i.e. 14,0616 mg sGnRH-Gly-Cys) was added to the PPD. The solution was mixed for 2 hours at room temperature under nitrogen and the free thiol content assessed throughout. The conjugate was dialysed overnight against distilled water in benzyolated cellulose tubing at 12°C then stored at -20°C until required.

b) Free Thiol Assay

Free thiol content of the conjugate was estimated using the reagent 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and standards of reduced glutathione. A scaled-down micro-titre plate version of Ellman's assay was used in which 2 μl 10mM DTNB and 250 μl PBS (pH8) were added to 50 μl sample and the absorption at 405 nm determined after 10 minutes.

c) Rainbow trout and immunisation regime

One hundred and thirty maturing rainbow trout (length ≃ 30 cm) were individually marked using various combinations of freeze brands. The length and weight of each fish were noted and a 600 μl blood sample was taken to determine preimmunisation serum antibody levels. In addition, serum steroid levels were assayed to determine the sex of the fish. The fish were divided into two groups and received either sGnRH-Gly-Cys-PPD or saline administered intraperitoneally in Freund's Complete Adjuvant (FCA). The treated and control groups were divided into males and females. The males were maintained at ambient light and temperature. The females were further subdivided into two groups: one into ambient light and temperature (fluctuating but at or below 14°C) and the other in ambient light and 14°C. The fish in the latter group were used to assess the effect of temperature on the anti-sGnRH-Gly-Cys antibody titres and any resulting differences in fish maturation. Throughout the experiment, at four weekly intervals, individually branded fish were weighed, measured and bled. Sera were assayed for anti-sGnRH-Gly-Cys antibody by ELISA (using sGnRH-Gly-Cys-BSA) and steroid hormone 17-β oestradiol and/or 11 ketotestosterone by RIA.

d) Steroid analysis

The concentration of 17-β oestradiol (E2) and 11-ketotestosterone (11-KT) in sera of maturing fish were determined by radioimmunoassays. In this assay

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radioactively labelled steroid binds to a limited amount of steroid-specific antibody, and this interaction is partially inhibited by the addition of unlabelled steroid.

e) Optimisation of anti-sGnRH-Gly-Cys ELISA

Salmonid GnRH-Gly-Cys was conjugated to Bovine Serum Albumin (BSA) for use in ELISA. Ten mg BSA in 0.5 ml Buffer A plus 1 mg GMBS in 5 µl DMF were combined and eventually conjugated to 6.246 mg sGnRH-Gly-Cys. ELISA checkerboard assays enabled the choice of a suitable sGnRH-Gly-Cys-BSA coating concentration for the anti-sGnRH-Gly-Cys ELISA in the maturation experiment.

Results

i) Anti-sGnRH-Gly-Cys antibody titres

The anti-sGnRH-Gly-Cys antibody titres 8 weeks post-immunisation with sGnRH-Gly-Cys-PPD (20 μg sGnRH-Gly-Cys/fish, i.p. in FCA) have increased relative to the control group as shown in Table 1 (males and females).

Table 1: Mean anti-sGnRH-Gly-Cys antibody titres (log_2 ± SE)

	4 weeks post immunisation	8 weeks post immunisation
sGnRH-Gly-Cys-PPD Group, n = 65	1.42 ± 0.17	4.33 ± 0.37
Control Group, n = 65	1.03 ± 0.02	1.20 ± 0.08

ii) Serum levels of 17-β oestradiol during the maturation of rainbow trout : effect of immunisation with sGnRH-Gly-Cys-PPD

Serum levels of 17-β oestradiol continued to increase in maturing female trout at the same rate in both the treated and control groups until 4 weeks post-immunisation, as shown in Table 2. By 8 weeks post-immunisation, the level of 17-β oestradiol in the control group continued to increase at the same rate. However, the sGnRH-Gly-Cys-PPD group of maturing females had significantly lower 17-β oestradiol levels than the control maturing females ($p<0.05$:t-test) at 8 weeks post-immunisation.

Table 2: Mean serum levels of 17-β oestradiol (ng/ml ± SE) during the maturation of rainbow trout and effect of immunisation with sGnRHa-PPD

	Before immunisation	4 weeks post immunisation	8 weeks post immunisation
treated group	7.35 ± 0.77 n = 26	16.85 ± 1.57 n = 23	21.04 ± 2.29 n = 23
Control Group	5.85 ± 0.39 n = 23	16.36 ± 1.33 n = 21	28.38 ± 2.61 n = 18

iii) Correlation between antibody titres and steroid levels.

It is apparent that the increase in antibody titres after immunisation with sGnRH-Gly-Cys-PPD correlates with a significantly lower increase in serum 17-β oestradiol levels in maturing rainbow trout. The data show that the pGnRH-Gly-Cys-PPD is active in delaying the onset to sexual maturity in female rainbow trout.

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Claims

1. An analogue of piscine GnRH having the amino acid sequence:

pGlu-His-Trp-Ser-Tyr-W-Trp-Leu-Arg-Pro-Gly-X-Y-Z

wherein W represents Gly or a D-amino acid,

X represents a bond, or one or more amino acids which may be the same or different,

Y represents Cys or Tyr or is absent, and

Z is absent or represents one or more amino acids which may be the same or different or a continuous or discontinuous peptide chain, the or each discontinuity being a non-α amino acid polyfunctional linker moiety or a retro-inverso amino acid with the proviso that when W is Gly and X and Y are absent Z is present.

2. An analogue as claimed in claim 1 wherein W is Gly.

3. An analogue as claimed in claim 1 or claim 2 wherein Y is Cys.

4. An analogue as claimed in any one of the preceding claims wherein X is one or more glycine residues.

5. An analogue as claimed in any one of claims 1 to 3 wherein X or Z comprises at least one T-cell epitope.

6. An analogue as claimed in any one of the preceding claims wherein Z comprises a retro-inverso amino acid.

7. An analogue as claimed in claim 6 having the formula:

$$A_1 - B - A_2$$

wherein B is a retro-inverso amino acid and A_1 and A_2 are the same or different and have the sequence:

pGlu-His-Trp-Ser-Tyr-W-Trp-Leu-Arg-Pro-Gly-X-Y-Z'

in which W, X and Y are as defined in claim 1 and Z' is

the same as Z defined in claim 1 except that it does not include a retro-inverso amino acid.

8. An analogue as claimed in claim 1 having the sequence: pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Arg-Pro-Gly-Gly-Cys.

9. An analogue as claimed in any one of the preceding claims conjugated to a carrier molecule.

10. A vaccine comprising an analogue as claimed in any one of claims 1 to 8 or analogue conjugate as claimed in claim 9, together with a veterinarially acceptable excipient.

11. A kit for detecting anti-piscine GnRH antibodies in a sample, said kit comprising an analogue as claimed in any one of claims 1 to 8, or analogue conjugate as claimed in claim 9, label means and a solid support.

12. Use of an analogue as claimed in any one of claims 1 to 9 for the preparation of a medicament for slowing, halting or regressing sexual development of fish or for controlling fertility in fish.

13. A process for the manufacture of an analogue of piscine GnRH having the amino acid sequence:

pGlu-His-Trp-Ser-Tyr-W-Trp-Leu-Arg-Pro-Gly-X-Y-Z

wherein W represents Gly or a D-amino acid,

X represents a bond, or one or more amino acids which may be the same or different,

Y represents Cys or Tyr or is absent, and

Z is absent or represents one or more amino acids which may be the same or different or a continuous or discontinuous peptide chain, the or each discontinuity being a non-α amino acid polyfunctional linker moiety or a retro-inverso amino acid with the proviso that when W is Gly and X and Y are absent Z is

present, the process comprising the steps of coupling the residues using chemical, biological and/or recombinant techniques known per se and isolating the analogue.

14. DNA coding for an analogue as claimed in any one of claims 1 to 5 or claim 8 wherein the analogue is a continuous peptide chain.

15. An antibody or antigen binding fragment which specifically binds to an analogue as claimed in any one of claims 1 to 8.

16. A kit for detecting piscine GnRH in a sample, said kit comprising an antibody or antigen binding fragment as claimed in claim 15, label means and a solid support.

17. Use of an antibody or antigen binding fragment as claimed in claim 15 in the manufacture of a medicament for slowing, halting, or regressing sexual development of fish or for controlling fertility in fish.

18. A process for the manufacture of an antibody or antigen binding fragment as claimed in claim 15, which process comprises immunising a subject with an analogue as claimed in any one of claims 1 to 8 or an analogue conjugate as claimed in claim 9 and isolating the antibody formed or cells which produce the antibody.

19. Anti-idiotype antibodies which are specific to an antibody or antigen binding fragment as claimed in claim 15.

20. A method of immunising fish to piscine GnRH to slow, halt or regress sexual development, or control, limit, or eliminate fertility comprising the step of administering to the fish an effective amount of an analogue as claimed in any one of claims 1 to 8 or an

analogue conjugate as claimed in claim 9 or an antibody or antigen binding fragment as claimed in claim 15.

21. A method as claimed in claim 20 wherein the fish is immersed in water containing an analogue as claimed in any one of claims 1 to 8 or an analogue conjugate as claimed in claim 9 or an antibody or antigen binding fragment as claimed in claim 15.

22. A method as claimed in claim 20 wherein an analogue as claimed in any one of claims 1 to 8 or an analogue conjugate as claimed in claim 9 or an antibody or antigen binding fragment as claimed in claim 15 is administered orally.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/16; C07K7/20; C07K15/00; G01N33/74
A61K39/00; A61K39/395

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; G01N ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	EP,A,0 293 530 (PROTEUS BIOTECHNOLOGY LTD) 7 December 1988 see the whole document	1-4, 8-13, 15-18
A	FEBS LETTERS. vol. 214, no. 1, April 1987, AMSTERDAM NL pages 65 - 70; C.A.MORRISON: 'COMPUTER-AIDED DESIGN AND PHYSIOLOGICAL TESTING OF A LHRH ANALOGUE FOR 'ADJUVANT-FREE' IMMUNOCASTRATION' see the whole document	1-4, 8-13, 15-18
A	EP,A,0 181 236 (PITMAN-MOORE, INC.) 14 May 1986 see the whole document	1,6,7

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° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02 APRIL 1992	06.05.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	GROENENDIJK M.S.M.

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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A	WO,A,8 401 771 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 10 May 1984 see the whole document	1

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9200040 SA 55264

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 02/04/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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